Supporting online material
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Materials and Methods
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Materials and Methods

Statistical analysis: *P* values were calculated with the Student's t-test with two-tailed distribution and two-sample equal variance parameters.
Mice: $LRRK2^{-/-}$, backcrossed seven generations onto C57BL/6J background facilitated with genome scan, and WT littermates or C57BL/6J mice (Jackson Laboratory) were used between 6 and 8 weeks of age. All experiments were performed in accordance with the NIAID Animal Care and Use Committee.
Cell culture and reagents: THP1 and HEK 293T cells were purchased from ATCC. Lipopolysaccharides (LPS), cyclosporine A, phorbol 12-myristate 13-acetate (PMA) and FK506 were purchased from Sigma, Pam3CSK4, muramyl dipeptide (MDP), polyI:C, zymosan and *E. coli* ssDNA were from InvivoGen. Ionomycin was purchased from Invitrogen. ELISA kits for IL-12 (p40), IL-2, IL-6, IL-1β, and TNF-α were from BD Biosciences. Mouse primary $CD4^+$ T cells were isolated from spleen and lymph nodes from WT C57BL/6J mice using mouse CD4 beads from Miltenyi.
Plasmids: pCMV-2XMyc-LRRK2, WT and kinase dead version (KD) expressing mycLRRK2 were kind gifts from Dr. Mark Cookson. NFAT1-GFP, caNFAT1-GFP, HA-NFAT2, HA-NFAT3, HA-NFAT4 were from Addgene. NFAT, NF-κB luciferase constructs and renilla luciferase construct (pRL-TK) were from Dr. Nicolas Bidere. The T2397 LRRK2 construct was generated with site-directed point mutagenesis, and confirmed with Sanger sequencing.
Antibodies: Anti-PSMD11 antibody was from Novus. Anti-Myc and anti-GFP antibodies were from Roche. Anti-HA antibody was from Sigma, anti-IQGAP1, anti-Hsp90, anti-PARP, anti-NFAT1 and anti-TNPO1 antibodies were from BD biosciences. Anti-CSE1L, anti-PPP2RA1 and anti-KNPB1 antibodies were from Bethyl Laboratories. Anti-mouse NFAT1 was from Santa Cruz. Anti-phospho-IκBα, phospho-ERK1/2, phospho-JNK and phospho-p38 antibodies were from Cell Signaling. LRRK2 antibodies were from Novus and Epitomics.
Luciferase assay: HEK 293T cells were transfected with luciferase reporter plasmids using ExGen 500. Cells were stimulated with PMA and ionomycin or human TNF-α for 6 hours. Luciferase activities were measured using the dual-luciferase kit (Promega).
qRT-PCR: Total RNA was isolated with RNeasy kit (Qiagen). cDNA was synthesized using SuperScript RT III (Invitrogen). qRT-PCR was performed using SYBR Green PCR mix (Applied Biosystems) with the following primers: Mouse LRRK2 forward primer, 5-CAGCTTCAGAAGGGACAAGG-3; reverse primer, AAGGCTGCGTTCTCAGGATA. Actin forward primer, AGCCATGTACGTAGCCATCC; reverse primer, CTCTCAGCTGTGGTGGTGAA.
BMDM and BMDC culture: BMDM and BMDC were cultured as described (*31*).
Cytosol-Nuclear fractionation: Transfected HEK293T cells or mouse BMDM cells

were treated with ionomycin or zymosan for 30 minutes, and cytosol and nuclear fractions were prepared as previously described (*32*).

Immunoprecipitation: Transfected HEK293T cells or THP1 cells were lysed in TNT buffer (50 mM Tris-HCl, 2 mM EDTA, 150 mM NaCl, 1% NP-40) with Protease Complete (Roche) for 30 min. Lysates were spun at 10,000xg for 10 min and precleared with Protein G Dynabeads for 30 minutes. Supernatant was incubated with antibodies for 1~2 hours before Protein G Dynabeads were added for 30 minutes. Protein G Dynabeads were washed for 5 times and eluted with SDS sample buffer.

Immunoblotting and quantification: Immunoblotting was performed with a standard protocol, except that IRDye-conjugated secondary antibodies were used for detection. Fluorescence signals were measured using Infrared fluorescence detection (LI-COR) to quantify protein levels.

Immunoprecipitation-RNA: Detection of NRON in LRRK2 complex was done following the RIP immunoprecipitation procedure (Millipore). NRON was detected by one-step RT-PCR (Qiagen) with NRON Forward primer 5'-CAGTAAAGGAGCAGTAGTGGAAACAG and Reverse primer 5'-TGGGGGGAGCGAATGGCATCGGGAAC.

DSS induced colitis: WT and LRRK2$^{-/-}$ mice received 3% (w/v) DSS (MP Biomedicals, m.w. 36,000 – 50,000) in their drinking water ad libitum for 8 days. Body weight and stool were monitored daily starting from treatment. For CsA treatment, CsA was dissolved in olive oil at 10 mg ml^{-1}, and 100 ml CsA solution or olive oil alone was intraperitoneally injected per mouse daily. At the end point, sera and colons were collected after mice were euthanized. Colons were fixed in 4% formaldehyde and sections were subjected to hematoxylin and eosin staining. Histological scoring was conducted by two blinded pathologists with the scoring criteria as described (*33*). NFAT1 immunohistochemistry (IHC) was performed by Histoserv.

Cycloheximide (CHX) chase assay: 293T cells were transfected with indicated plasmid. 24 hours later, cells were treated with CHX (100 μg ml^{-1}) for various periods of time, then were lysed and analyzed by immunoblot.

Ionomycin pulse-chase assay: 293T cells were transfected with indicated plasmids. 24 hours post transfection, cells were treated 1 μM ionomycin for 30 minutes. Ionomycin was washed away and fresh medium was added and cells were harvested at different time points and lysed.

Human peripheral blood: Normal human peripheral blood specimens were from the NIH blood bank. B cells were isolated using panB isolation kit (Invitrogen), and genomic DNA was purified using DNeasy Kit (Qiagen). Genomic DNA sequence of LRRK2 from 144657nt to 144947nt was determined by Sanger sequencing, and cell lysates were prepared from B cells homozygous for Met2397 or Thr2397 in RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP-40, and complete protease inhibitors).

Bone-marrow transfer: WT C57BL/6J received 10x10^6 bone marrow cells via tail vein injection after being subjected to sublethal gamma radiation (900 Rads). 6 weeks after transfer, mice were tested for DSS induced colitis.

Immunohistochemistry: NFAT1 immunohistochemistry was performed by Histoserv with NFAT1 antibody at 1:50 dilution.

Cytokine production and ELISA: BMDMs, plated in 96 well plates, were treated with various stimulators at indicated concentration for 18 hours, and supernatants were harvest. For IL-1β induction, BMDMs were first primed with 25 ng ml^{-1} LPS for 12 hours, and treated with 5 mM ATP for 3 hours. Supernatants were harvested. IL-12/p40, IL-6, TNF-α, and IL-1β ELISA were performed according to manufacturer's instructions (BD Bioscience).

NFAT1-GFP nuclear translocation: 293T cells stably transfected with Dox-inducible V5-LRRK2 were treated with 1 μg ml^{-1} Dox or DMSO for 24 hours, and transfected with NFAT1-GFP. 24 hours after transfection, cells were treated with 1 μM ionomycin for 60 minutes, and then fixed with 2% paraformaldehyde. Nuclei were visualized with Hoechst. For caNFAT1-GFP confocal imaging, cells were grown on glass chamber slides and living cells were imaged.

Microscopy: Leica SP5 confocal microscope was used to visualize GFP. Leica Episcope equipped with Axiocam was used to visualize histology slides.

Reference:

31. I. Zanoni *et al*., *Nature* **460**, 264 (Jul 9, 2009).
32. F. Wan *et al*., *Cell* **131**, 927 (Nov 30, 2007).
33. J. R. Maxwell *et al*., *Current protocols in pharmacology* **5**, 1 (Dec, 2009).

Supplementary Figures

Fig. S1

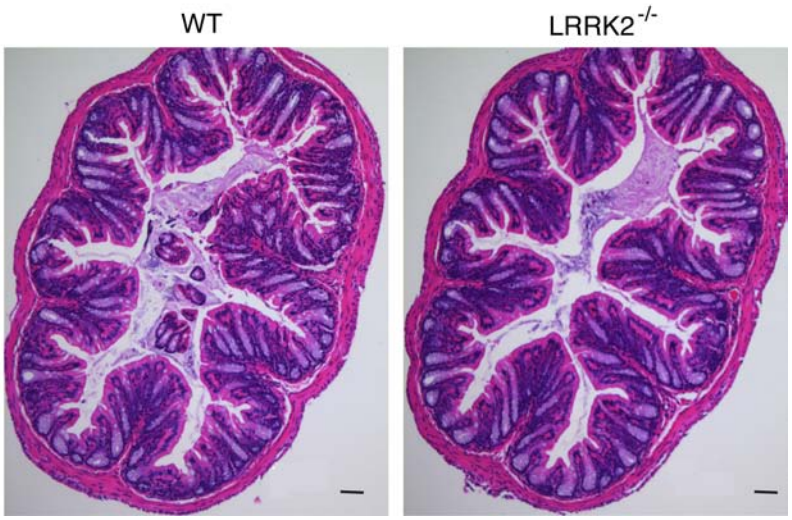

Fig. S1. Representative images of hematoxylin and eosin (H&E) staining of colon sections from unmanipulated wild type (WT) and LRRK2$^{-/-}$ mice. Scale bar, 100 μm.

Fig. S2

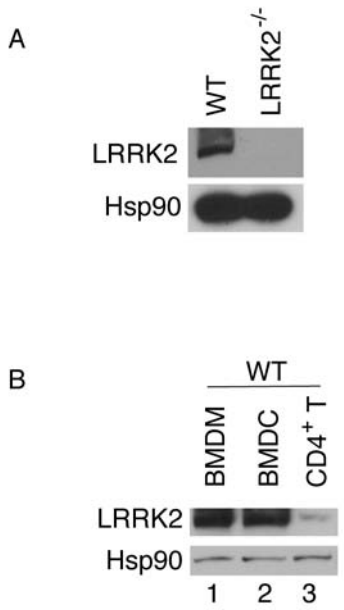

Fig. S2. Expression of LRRK2. (A), LRRK2 detection in bone marrow derived macrophage cells (BMDM) from WT and LRRK2$^{-/-}$ mice. (B), LRRK2 expression in BMDM cells, bone marrow dendritic cells (BMDC) and CD4^{+} T cells from WT mice detected by immunoblotting. Hsp90 was used as a loading control for cell lysates.

Fig. S3

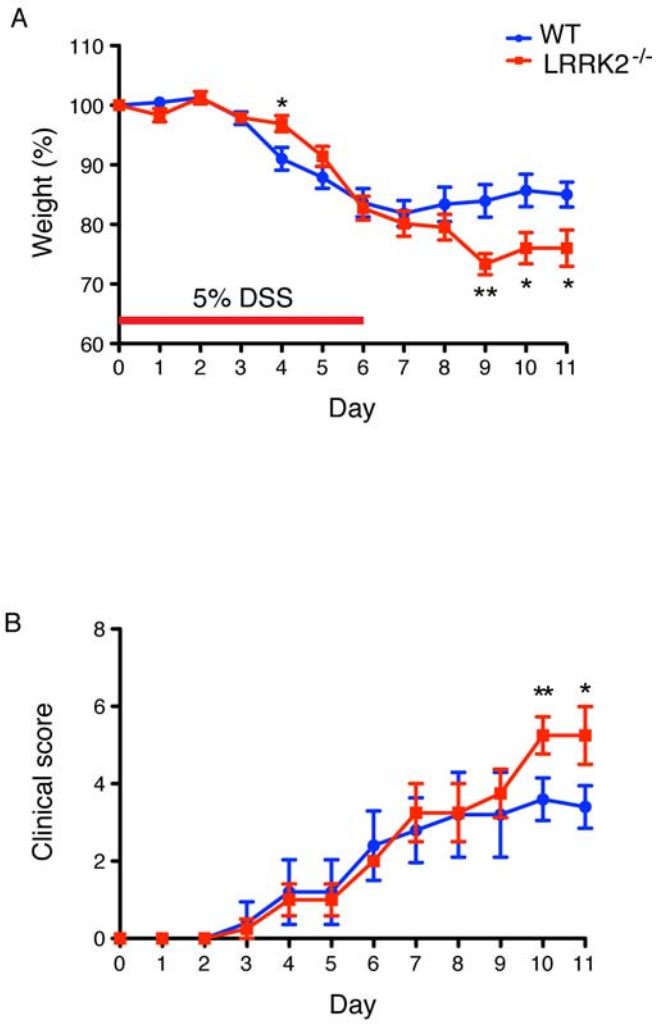

Fig. S3. LRRK2 deficiency exacerbates DSS colitis in mice. Mean body weight as a percent of starting weight (A) and mean clinical scores (B) of WT (blue, n=5) and LRRK2$^{-/-}$ (red, n=5) mice. Mice were treated with 5% DSS for 6 days followed by regular drinking water. * $P < 0.05$. ** $P < 0.02$. Error bars represent standard errors of the mean (SEM). The experiment was repeated twice.

Fig. S4

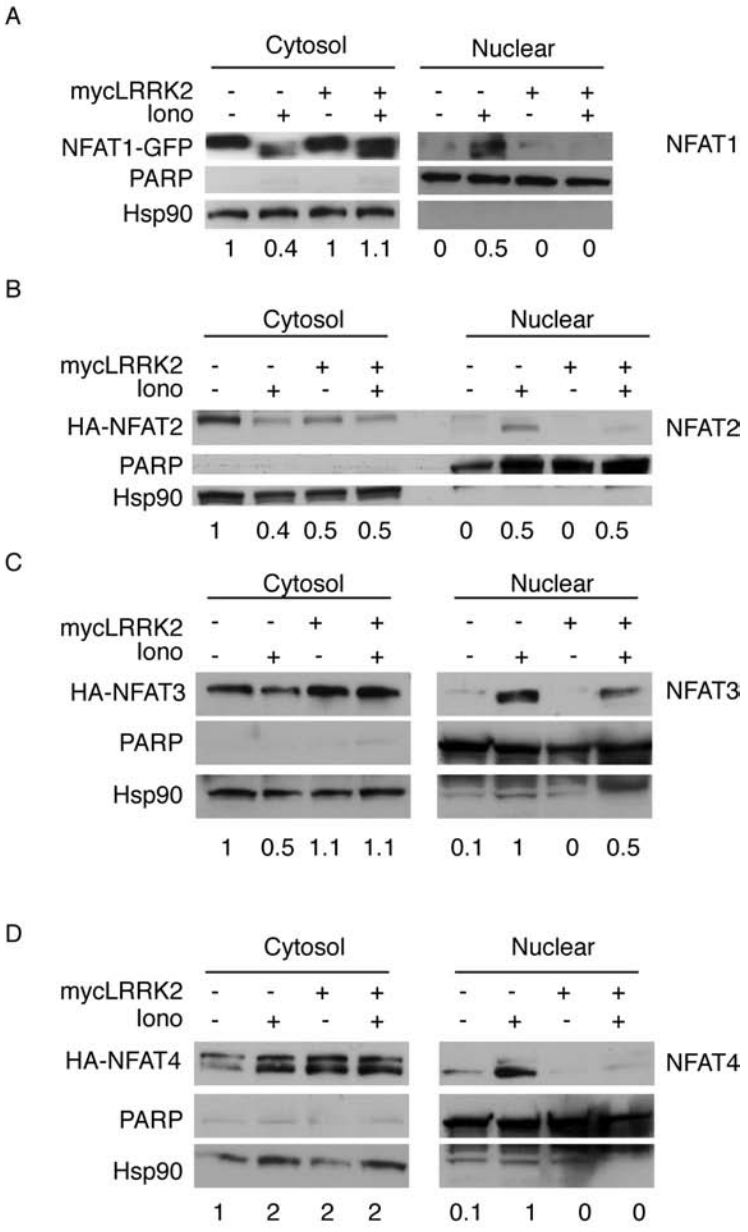

Fig. S4. LRRK2 inhibits nuclear translocation of NFAT. Immunoblotting of NFAT1-GFP (A), HA-NFAT2 (B), HA-NFAT3 (C) and HA-NFAT4 (D) in 293T cells transfected with individual NFAT plasmid and vector control or myc-LRRK2 in cytosolic vs nuclear fractions after 1 μM ionomycin treatment for 30 min. Hsp90 and PARP were used as controls for the purity of cytosolic and nuclear fractions, respectively. The relative intensity of NFAT bands is indicated at the bottom of the blots.

Fig. S5

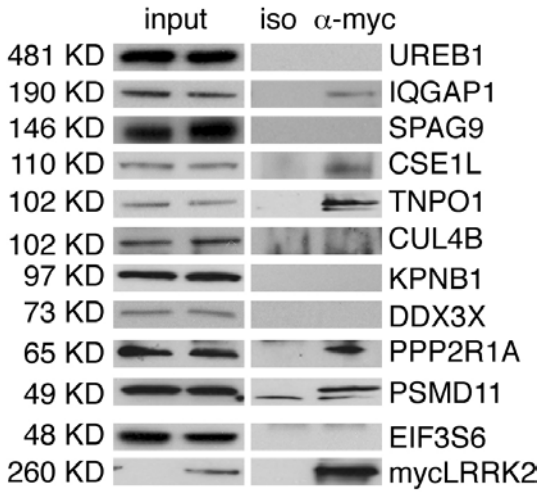

Fig. S5. Co-immunprecipitation of individual proteins in the NRON complex with myc-LRRK2. Immunoprecipitation/immunoblotting, as indicated, in 293T overexpressing myc-LRRK2. Iso, isotype control for immunoprecipitation. Sizes of proteins are indicated on the left.

Fig. S6

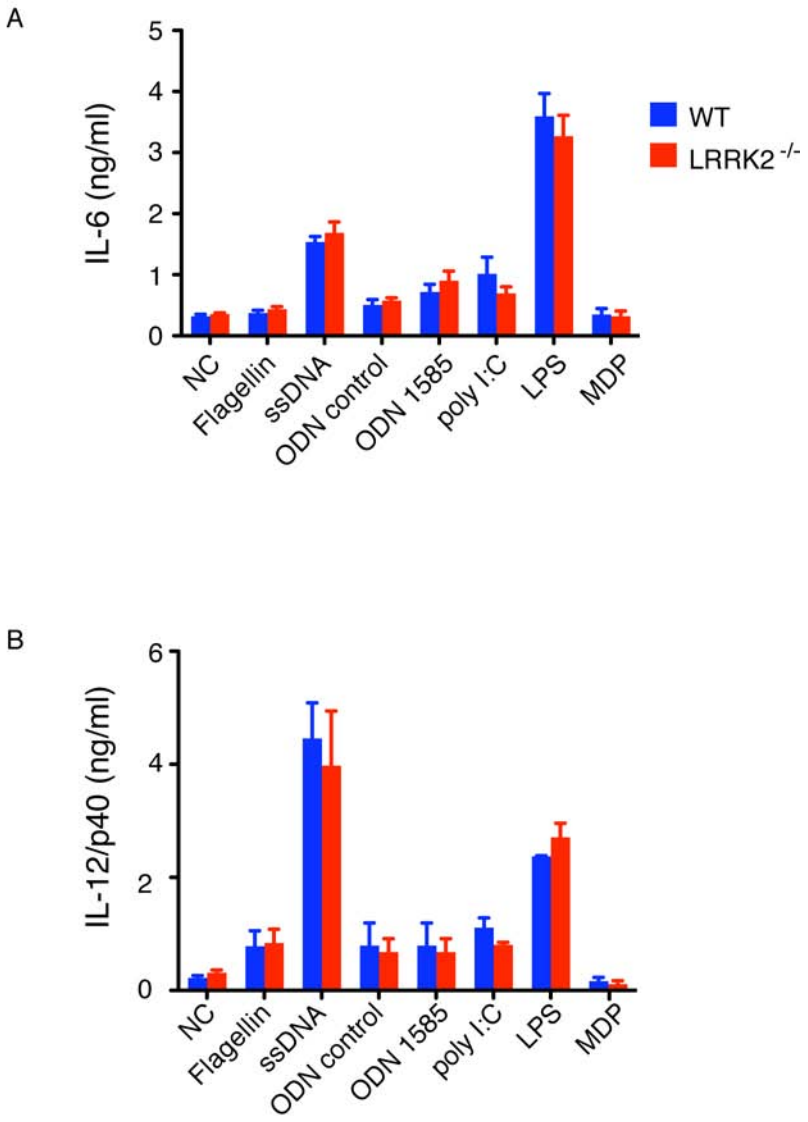

Fig. S6. Cytokine production by WT and LRRK2$^{-/-}$ BMDMs. IL-6 and IL-12/p40 production by BMDMs stimulated for 18 hours with 5 μg ml^{-1} flagellin, 10 μg ml^{-1} *E. coli* single stranded DNA (ssDNA), 2.5 μM oligodeoxynucleotides (ODN) control, 2.5 μM ODN 1585, 2.5 μM polyinosinic-polycytidylic acid (poly I:C), 10 μg ml^{-1} lipopolysaccharide (LPS) and 10 μM muramyl dipeptide (MDP). Data are means of triplicate wells with SEM.

Fig. S7

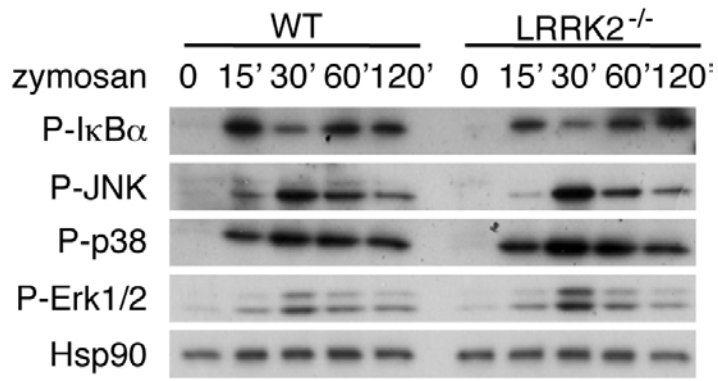

Fig. S7. LRRK2 deficiency does not affect NF-κB and MAPK activation by zymosan. WT and LRRK2$^{-/-}$ BMDM cells were treated with 100 μg ml^{-1} zymosan at designated time points in minutes and lysed. Lysates were immunoblotted for p-IκBα, p-JNK, p-Erk1/2 and p-p38. Hsp90 was used as a loading control for cell lysates.